AD_____

AWARD NUMBER: W81XWH-13-1-0298

TITLE: Stress Altered Stem Cells with Decellularized Allograft to Improve Rate of Nerve Regeneration

PRINCIPAL INVESTIGATOR: Dr. Charles A. Vacanti

CONTRACTING ORGANIZATION: BRIGHAM AND WOMEN'S HOSPITAL
Boston, MA 02115

REPORT DATE: October 2014

TYPE OF REPORT: Annual Technical Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE October 2014	2. REPORT TYPE Annual	3. DATES COVERED 10 Sep 2013 - 9 Sep 2014

4. TITLE AND SUBTITLE
Stress Altered Stem Cells with Decellularized Allograft to Improve Rate of Nerve Regeneration

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0298

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Charles A. Vacanti

E-Mail: cvacanti@partners.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Brigham and Women's Hospital
Department of Anesthesia
75 Francis Street
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The research detailed in this annual report is to study how to improve the rate of peripheral nerve regeneration. The slow rate of nerve regeneration in limbs results in poor prognosis for patients suffering from severe injuries, leading to muscle impairment, and in extreme cases, atrophy. For our research, we will study the rate at which nerves regenerate in a rat model. We will excise the peripheral nerve, and study how to modulate the nerve regeneration through the use of a decellularized nerve graft and stress altered cells (SACs), a cell type we have identified that show stem-cell like qualities after undergoing physical and chemical stresses.

15. SUBJECT TERMS
Peripheral Nerve Repair, Nerve Injury, Decellularized Allograft, Neural Regeneration, Stress Altered Cells, Rat Peripheral Nerve Injury Model

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	19	USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The research detailed in this annual report is to study how to improve the rate of peripheral nerve regeneration. The slow rate of nerve regeneration in limbs results in poor prognosis for patients suffering from severe injuries, leading to muscle impairment, and in extreme cases, atrophy. For our research, we will study the rate at which nerves regenerate in a rat model. We will excise the peripheral nerve, and study how to modulate the nerve regeneration through the use of a decellularized nerve graft and stress altered cells (SACs), a cell type we have identified that show stem-cell like qualities after undergoing physical and chemical stresses.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Peripheral Nerve Repair, Nerve Injury, Decellularized Allograft, Neural Regeneration, Stress Altered Cells, Rat Peripheral Nerve Injury Model

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The Overall summary of our project goals are stated as follows:

1) Identification of proper tissue source and cell culture techniques for creating Stress Altered Cells (SACs)
2) Evaluate the potentials of SACs through immunohistochemistry, FACS Analysis, and Differentiation assays
3) Harvest and Decellularize rat nerves for use as an allograft, followed by subsequent analysis and characterization of a cell-seeded allograft
4) Creation of a peripheral nerve defect in the sciatic nerve of the rat, along with neurological testing pre-graft implantation
5) Implantation of cell-seeded allografts into rats with sciatic nerve defects, along with neurological testing post-graft implantation

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the

project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We are currently working on Goal 1 of our project, and have made progress in identifying the cells to harvest for generation of SACs. We have selected CD45+ cells as the appropriate cells for harvest. These cells are primarily leukocytes, and we have isolated them from rat spleens. Due to the lymphocyte's fully differentiated hematopoietic lineage, we can safely assume that they are cells that do not exhibit stem-cell like qualities. We have also found methods of isolating the correct cells, and have confirmed the results through fluorescence-activated cell sorting (FACS).

When we began the project, we began by trying to find the proper source of cells and method of creating the SACs. When we originally discovered the SACs in mice, we had performed the treatment to mouse CD45+ cells (leukocytes) isolated from the spleen. We decided to use the same type of cells of cells from the Lewis rats that we harvest tissue from. The cells we harvested were initially sorted by using Mammalian Lympholyte to separate lymphocytes from the spleen contents. However, when we tried to confirm during the FACS process, it was found that a majority of the cells isolated from the rat spleen were red blood cells. We have found that red blood cells in the presence of CD45+ cells had an inhibitory effect on the creation of the SACs. We further refined the isolation technique, and used Lympholyte-R in conjunction with a red blood cell lysis buffer. This removed a substantial amount of the red blood cells, and improved the fidelity of our FACS analysis. During the procedure, we additionally found 3 distinct groups that were all CD45+. Currently, we are sorting for the largest population of CD45+ cells for the stress altering treatment, though it is possible for us to select the ancillary populations for treatment and identify if they are better for treatment.

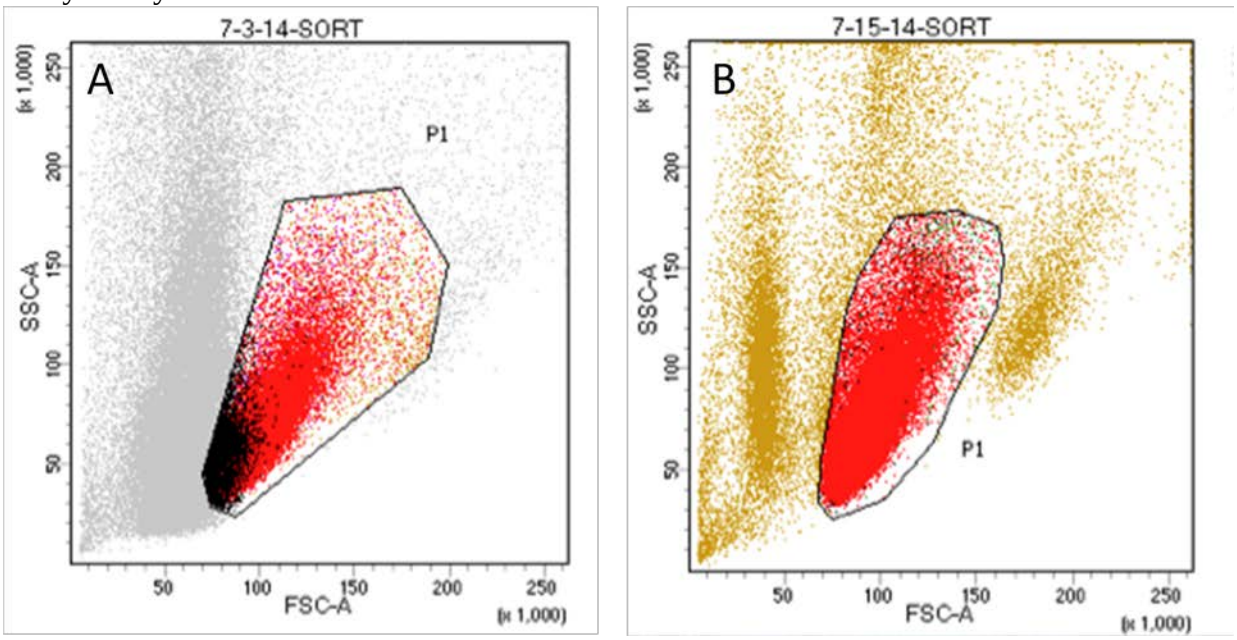


Figure 1: The FACS analysis of various isolation methods. Figure 1A shows the isolation using only Lympholyte, Figure 1B shows Lympholyte in addition to the lysis buffer. With the addition of lysis buffer, you can see new groups emerge from the separation

After confirming the method of cell purification, we began optimizing the method of stress-altering the cells. Prior to this, our experience with the mouse SACs led us to try the treatment we optimized for them. We exposed the cells to a Hank's Balanced Salt Solution(HBSS) titrated to a pH of 5.7 for 30 minutes to stress the cells, but we found that we were getting poor response from the rat cells in this environment, and they would not create the SACs. We re-optimized the treatment for the rat cells, and found that treatment in HBSS titrated to a pH of 5.4, in conjunction with mechanical trituration through a fire-polished pipette resulted in the rat cells that began clustering in a manner similar to SACs.

We've also found that the concentration of cells treated was important to the formation of SACs. While the presence of other cells can impede the production of SACs (such as the presence of red blood cells in lympholyte) too few cells can result in a lack of SAC formation. However, with too many cells, isolating the SAC clusters becomes difficult, and we believe differentiation into the proper cell type may be compromised by the presence of single cells.

In addition to the cell identification and evaluation work performed in our lab, the sub-awardee AxoGen has harvested and decellularized sciatic nerve segments from Sprague-Dawley rats. These nerves are now ready to be used in the cell seeding experiments.

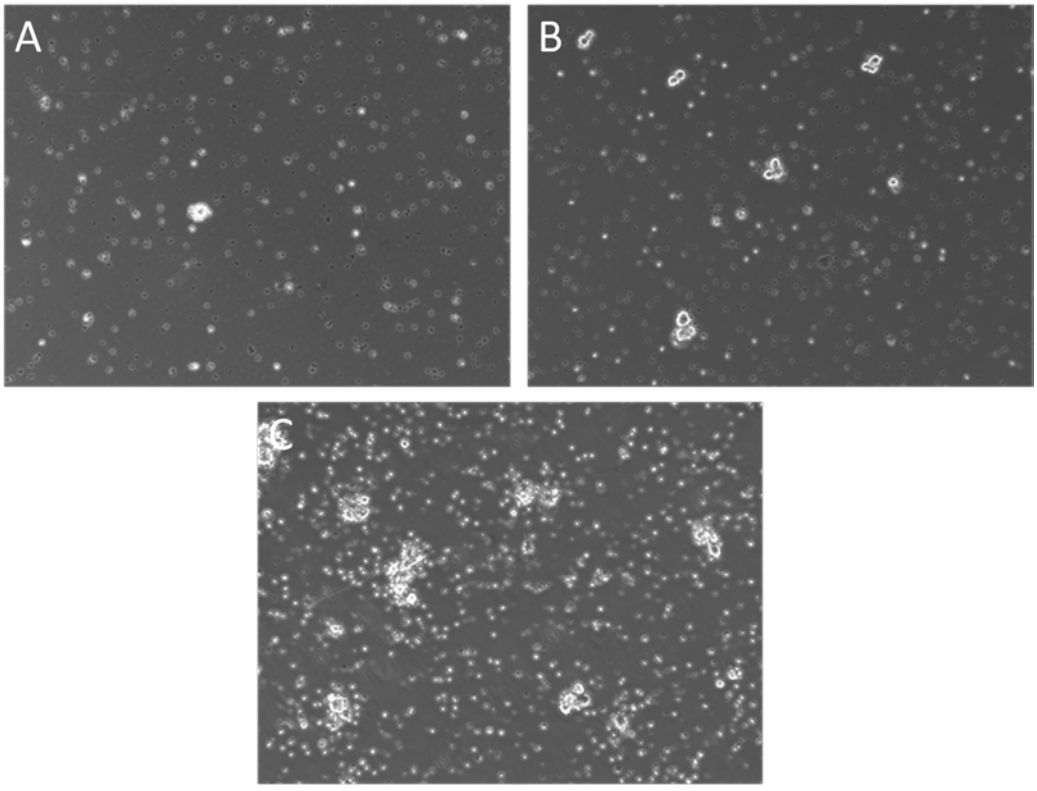


Figure 2: Images of sphere formation at various cell densities. 2A shows minimal SAC formation at 2.5E5 cells per 3 ml, 2B shows moderate sphere formation at 1E6 cells in 3 mL, and 2C shows a large number of clusters forming from 1E7 cells in 3 mL. It is easier to select the SACs in the medium concentrations, but quality improves at high concentrations.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The next steps to perform are the *in vitro* cell seeding into the decellularized scaffold made by our collaborators at AxoGen, Inc. The timing for implantation is important after excision of the native nerve, so it is important to know a list of variables for cell seeding prior to implantation; cell density, time for differentiation, media ingredients are among important variables to consider.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

No significant changes in objectives and scope

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Due to the variable nature of SAC generation, we have had trouble generating the SAC cells consistently, though we believe we have solved the problem through the increased stress (Trituration in Acidic buffer) as well as the concentration for proper SAC generation. Our goal is to identify the method of seeding cells into the scaffold and direct growth through the length of the scaffold. Due to the difficult nature of differentiation, we need to ensure that the environment is conducive for neural differentiation as well as cells that preferentially adhere to the scaffold

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

No significant changes to expenditures

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No Human subjects in use

Significant changes in use or care of vertebrate animals.

No significant change in use or case of vertebrate animals

Significant changes in use of biohazards and/or select agents

No biohazard agents used

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title;*

journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Name: Dr. Charles A. Vacanti
Project Role: Principal Investigator
Research identifier:
Nearest person month worked: 9 Months
Contribution to Project: Discussion and review of produced data

Name: Dr. Koji Kojima
Project Role: Co Principal Investigator
Research Identifier:
Nearest person month worked: 9 months
Contribution to Project: Tissue Harvest, culture, and creation of SAC cells. Work done on Hypoxia/Anoxia studies

Name: Siddharth Ramshankar
Project Role: Research Technician
Research Identifier:
Nearest person month worked: 9 months
Contribution to project: Harvest, Culture, and creation of SAC cells. Work done on acid and trituration SAC generation.

Name: Curt Deister (AxoGen)
Project Role: Sub-award PI
Nearest person month worked: 3 months
Contribution to Project: Acquisition of animal tissue, processing of animal tissue and review of literature on seeding of decellularized grafts

Name: Curtis Thompson (AxoGen)
Project Role: Sub-award laboratory technician
Nearest person month worked: 3 months
Contribution to Project: Acquisition of animal tissue and processing of animal tissue

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

AxoGen, Inc – Collaboration
Alachua, Florida
Collaboration in creating decellularized tissue grafts necessary for cell seeding

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Stress Altered Stem Cells Combined with Decellularized Nerve Allografts to Improve Rate of Nerve Regeneration

OR120208 / W81XWH-13-1-0298

PI: Charles A. Vacanti **Orgs:** Brigham & Womens Hospital, AxoGen Inc. **Award Amount:** $710,212

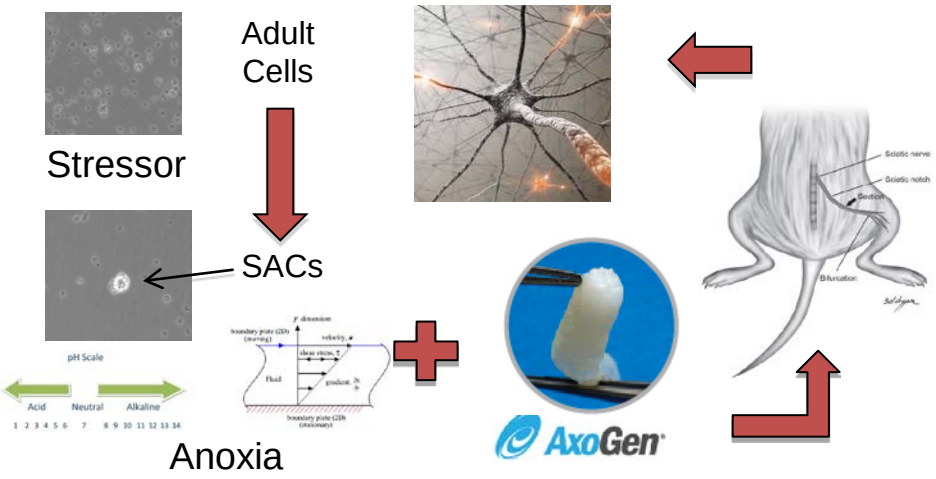
Stress-Activated Pluripotent Cells (SACs) with No Genetic / Chemical Manipulation Implanted with Commercially Available Allograft in Rat Sciatic Injury

Specific Aim 1: Induce stress altered stem cells and compare different stresses and combination of stresses to evaluate yield and potency.

Specific Aim 2: Develop seeding methods to deliver stress altered stem cells ex vivo with species-specific decellularized nerve scaffolds in a long graft rat sciatic model.

Specific Aim 3: Assess the rate and degree of functional nerve repair of decellularized scaffolds seeded with stress altered stem compared to controls.

Stresses to be Evaluated: pH, Mechanical Trituration, Anoxia



Accomplishments: Stress alteration experiments underway, studying more consistent methods by expanding the range of treatments.

Timeline and Cost

Description	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Cell Culture																								
Stress Alteration																								
Combine Stresses																								
Stress Analysis																								
Process Graft																								
Cell Seeding																								
Characterize Graft																								
Implantation																								
Analysis & Close-Out																								
Estimated $	CY13: $75K								CY14: $325K											CY15: $100K				

Updated: (V4: 8/9/2014)

Goals/Milestones

CY13 Goal – Complete Initial Cell Culture & stress Alteration
- ☑ Receive IACUC & ACURO approvals
- ☑ Set up Cell Culture Systems
- ☑ Begin Stress Alteration

CY14 Goals – Characterize Grafts & Begin Implantation
- ☑ Combine multiple stresses to optimize yield and potency
- ☐ Evaluate cell seeding and graft processing
- ☐ Begin graft implantation with optimized stress altered cells

CY15 Goal – Complete Implantation & Analyze Intervention
- ☐ Complete surgical interventions
- ☐ Analyze rates of nerve repair compared to control

Comments/Challenges/Issues/Concerns: None Currently

Budget Expenditure to Date

Projected Expenditure: $333K to date

Actual Expenditure: $183.4K to date